EXHIBIT "A"

II. SUMMARY OF THE INVENTION

Accordingly, the present invention provides improved sheath and collector systems for sorting of sperm cells to determine their sex through a flow cytometer. The sheath fluid as typically used in a flow cytometer is replaced with a fluid which minimizes the stress on the sperm cells as they are sorted. Furthermore, the collection system is improved to minimize both the physical and chemical stress to which the sperm cells are subjected. Various techniques and substances are represented but as those skilled in the art will readily understand, various combinations and permutations can be used in the manner which may be optimized for performance based in the species, goals and other parameters involved in a specific processing application.

An object of the invention is thus to achieve better sorting for substances such as sperm cells. A goal is to minimize the impact the sorting function itself has on the cells or other sensitive items which may be sorted. A particular goal is to minimize the impact the sheath fluid imposes upon the cells and to potentially provide a sheath fluid which affirmatively acts to assist the cells in handling the various stresses involved. A parallel goal is to provide substances and techniques which are especially suited for sperm cells in general, for bovine sperm cells, for equine sperm cells, and for the separation of such sperm cells into X- and Y-chromosome bearing components. Similarly a goal is to minimize the impacts that the collection phase (e.g., after sorting) has upon the cells and to minimize the physical impact as well as chemical impacts on such sex sorted sperm cells. Thus a goal is to achieve as unaffected a sorted result as possible.

Another object of the invention is to achieve low dose, sorted insemination on levels and with success rates which are comparable to those of the typical unsexed, high dose artificial insemination. Thus the prior goals of minimizing the stress or potential damage upon the sperm cells is important. Sorting in a manner which affords both high speed and low stress sorting, and which is especially adapted for sperm cell sorting in a low dose context is an important goal as well. The goals of

providing sheath and other fluids which do not negatively affect the fertility of the sperm and which are compatible with artificial insemination are also important.

Naturally further objects of the invention are disclosed throughout other areas of the specification and claims.

III. BRIEF DESCRIPTION OF DRAWINGS.

Figure 1 is a schematic diagram of a sorter system according to the present invention.

Figure number 2 is a diagram of the entrained cells in the free fall area of a typical flow cytometer.

Figure 3 is a conceptual diagram showing differences as they roughly appear as a result of the present invention.

Figure number 4 is a diagram of the sorted cell stream as they are collected in the landing zone area.

IV. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT.

As will be seen, the basic concepts of the present invention can be combined and embodied in a variety of ways. The invention involves both improved flow cytometer systems as well as systems for the creation of sex-specific sperm samples which may be used in artificial insemination and the animals produced by such techniques. Furthermore, the techniques are disclosed in a general fashion so that they may be applied to specific systems and applications once the general principals are understood. While device enhancements are disclosed it should be understood that these

been stained according to the Johnson technique, the light stimulation by laser exciter (11) is differentially determined by sensor (10) so that the existence or nonexistence of a charge on each drop (8) as it separates from stream (7) can be controlled by the flow cytometer. This control results in positively charged, negatively charged, and uncharged drops (8) based upon their content. As shown in Figure 2, certain drops are shown as deflected drops (16). These deflected drops (16) are those containing sperm cells (15) of the one or the other sex. They are then deposited in the appropriate collector (14) for later use.

One of the aspects of flow cytometry which is particularly important to its application for sperm sorting is the high speed operation of a flow cytometer. Advances have been particularly made by the flow cytometers available through Cytomation, Inc. under the MoFlo® trademark. These flow cytometers have increased sorting speeds extraordinarily and have thus made flow cytometry a technique which is likely to make feasible the commercial application of sperm sorting (among other commercial applications). They act to achieve high speed sorting, that is at a speed which is notably higher than those otherwise utilized. Specifically, Cytomation's MoFlo® flow cytometers act with oscillator frequencies of greater than about five kilohertz and more specifically can be operated in the 10 to 30 or even the 50 kilohertz ranges. Thus droplets are formed at very high frequencies and the cells contained within the sheath fluid environment can be emitted very rapidly from the nozzle (2). As a result, each of the components such as the nozzle (2) oscillator (6), and the like which make up and are part of a flow cytometer system result in a high speed cell sorter. In the application of a high speed cell sorter to the sorting of sperm cells, sorting at rates of greater than about 500 sorts per second is achieved. In fact, rates of sorting in the thousand and twelve hundred ranges have already been achieved through a high speed cell sorter. Importantly, it should be understood that the term "high speed" is a relative term such that as other advances in flow cytometry and specific applications are achieved, the aspect which is considered "high" may be varied or may remain absolute. In either definition, the general principle is that the sorting may occur at rates at which the parameters and physical characteristics of the flow cytometer are significant to the cells themselves when sorting particular cells such as sperm cells.

One aspect of high speed sorting which appears to come into play when sorting sperm cells is that of the pressures and other stresses to which the sperm cells are subjected within the flow cytometer. For instance, when operating at high speeds (and an alternative definition of "high speed"), flow cytometers can be operated at a pressure of 50 pounds per square inch and even 60 and higher pounds per square inch. These pressures may be considered high because they may result in effects upon the cells being sorted. The key as disclosed in the present invention for this facet is the fact that the stress thresholds of the particular cells are the determining factor. Additionally as further knowledge is gained it may be shown that the stress thresholds are a function of combined effects such as the particular species or the particular prior or subsequent handling of the cells. The key in this regard is that the stress imposed upon the cells can, in fact, alter their viability and their ability to achieve the desired result. In the pressure case, it may be that merely subjecting the sperm cells to a higher pressure as a result of the operation of the flow cytometer at that pressure may result in decreased performance of the cells. The present invention in one regard acts to minimize these stresses and thus results in greater efficacies as well as lower dosages as discussed later.

In considering the stress aspect of the cells, the present invention acts in a fashion which minimizes the stresses. These stresses can be minimized at any point in the over all cycle or process of collecting, sorting or even inseminating the animal. Importantly, the stress imposed by the handling of the cells within the flow cytometer appears significant for this application. In one embodiment of the invention, the sheath fluid is specifically selected so that it can serve in a coordinated fashion with both (or either) the pre-sort cell fluid environment or the post-sort cell fluid environment. While naturally it is possible to adjust either the pre- or post-sort fluids, in one embodiment the invention adjusts the sheath fluid (3) so that it imposes significantly less stress upon the cells than was previously accomplished. In one regard the invention is remarkable in that it removes the total focus from that of operation of the flow cytometer to a focus on handling and removing stress from the cells themselves. For instance, while it has been known to utilize fluids having a proper pH factor or osmoality, the present invention recognizes that there may be certain chemical compositions to which the cells may be hyper-responsive. These hyper-responsive chemical compositions may naturally vary based upon the cells or even the prior handling of the cells. Importantly at present it appears that for

sperm cells certain metabolic chemical compositions such as citrate seem to prevent unusually high stresses upon the cells. Thus, the hyper-responsive chemical compositions can be defined as those to which the cells are particularly responsive in the context of their functionality and the then-existing handling techniques. As to sperm cells it appears that metabolic compositions, specifically citrate constancy for bovine sperm cells and hepes buffer constancy for equine sperm cells may be very important. Thus the present invention acts to minimize the changes through the type of operation or the selection of substances which may act as a means for minimizing the changes which the cells experience.

For the sheath fluid, a substance is selected according to one embodiment of the invention so that it may be chemically coordinated to prevent minimal changes. Thus, by selecting the appropriate sheath fluid not only in context of flow cytometry parameters, but rather also in context of the cell parameters themselves, the changes experienced by the cells and the over all result of the sorting can be enhanced. This is shown conceptually in Figure 3. Figure 3 shows some type of chemical factor (such as citrate or other factors) as it may exist throughout the various phases of the process. For instance, the four phases shown might represent the following: phase I may represent the existence of the cells within the cell source (1), phase II might show the existence of the cells as they are sorted in the sheath fluid environment, phase III might show the cells as they are collected after sorting and phase IV might show the reconstituted cells in a storage medium after sorting. These four phases as shown for the prior art may experience vastly different chemical factor environments. As shown conceptually, however, in the present invention the cells may experience very little change, most notably the dip or drop experienced between phases I and II may be virtually absent. This is as a result of the selection of the appropriate sheath fluid as mentioned above. Thus, as a result of being subjected to an appropriate sheath fluid, the cells in the present invention may experience a much lower level of stress.

One of the potential generalities that may exist with respect to this phenomenon is the fact that certain chemical compositions may represent more hyper-responsive chemical compositions than others. While naturally this may vary based upon the species of sperm, the handling, or even the type

of cell involved, it appears that the viability of the cells for their intended purpose (here, artificial insemination) varies greatly, naturally or because of sorting or both, and so the cells exhibit a hyper-responsive character with respect to that chemical composition. By selecting certain metabolic chemical compositions, most notably citrates or chemicals which are within the citric acid cycle, great advances appear possible. Thus for the bovine sperm application, the sheath fluid (3) is selected and coordinated so that it presents about a 2.9 percent sodium citrate composition. Specifically, the 2.9 percent sodium citrate solution may be created as follows:

1. Place 29.0 grams of sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in a 1,000 ml volumetric flask
   a. Dissolve sodium citrate in ¾ of water batch, then add water to volume.
2. Add deionized or Nanopure water to make 1,000 ml final volume.
3. Transfer to bottles and autoclave at 15 lbs pressure (245°F) for at least 30 minutes
   a. Autoclave solution using conditions to minimize evaporation (loose cover)
   b. Be careful that water does not boil away.
4. Cool slowly at room temperature.
5. Store sealed in a 5°C cold room.

Further, for a sheath fluid, the sodium citrate solution may be filtered.

6. Filter with a .22 micron filter using aseptic techniques.

Interestingly, for equine sperm cells such a composition does not perform as well. Rather, it has been discovered that for equine sperm cells, a hepes buffered medium such as a hepes bovine gamete medium — particularly HBGM3 as previously created by J. J. Parrish for a bovine application — works well. This medium is discussed in the article "Capacitation of Bovine Sperm by Heparin", 38 Biology of Reproduction 1171 (1988) hereby incorporated by reference. Not only is this surprising because it is not the same type of substance as is utilized for bovine sperm, but the actual buffer, originally was developed for a bovine application. Thus in the equine application the sheath fluid is selected which contains the hepes buffer. This solution may have a pH at room temperature of about 7.54 (pH at 39°C = 7.4) with the following composition:

Chemical	Dry weight (g/500ml)
$CaCl_2$	0.145

KCl_2	0.115
$MgCl_2 \cdot 6H_2O$	0.004
$NaH_2PO_4 \cdot H_2O$	0.018
NaCl	2.525
NaPyruvate	0.011
Lactic Acid (60%)	1.84 ml
HEPES	4.765
$NaHCO_3$	0.420
BSA (fraction V)	3.0

One other aspect which may interplay in the present invention is the fact that the cells involved may experience unusual sensitivities. In one regard this may be due to the fact that sperm cells are in a class of cells which are non-repairing cells. That is, they do not have the ability to repair themselves and hence, they may need to be treated much more sensitively than is typical for flow cytometers or other handling equipment. Thus, it may be appropriate that the enhancement is particularly applicable when the flow cytometer acts to establish a source of sperm cells. Another potentially related aspect which may be unique to a class of cells such as sperm cells is the fact that their DNA is non-repairing, non-replicating, and non-transcribing. Either of these factors may come into play and so they may be relevant either individually or together. Thus, it may be that the teachings of the present invention apply to all gamete cells or even to viruses and the like which are non-repairing, non-translating, non-transcribing cells.

A separate aspect of the flow cytometer processing which may also be important is the fact of properly treating the cells both chemically and physically after they are sorted. As shown in figure 4, as the cells within drops (8) land in collector (14), it may be important that the container which makes up the collector be properly sized so that it acts as some means of avoiding an impact between the cells and the container itself. While it has been known to place an initial collector fluid (17) in the

bottom of the container to collect the cells so that they do not hit the bottom of the container, it appears that a simple widening of the container to address variations in stream presentation as well as the inevitable splashing due to the impact of the cells into the container can be used to enhance the result. In one regard this can act as a cushioning element so that cells which may be mechanically delicate, that is, they may break or be damaged by an impact can be treated appropriately. Thus when the cytometer source establishes cells which are physically delicate cells as the cells to be sorted, it may be important to provide some type of cushioning element such as a wide collection tube for which the opening width (18) serves to position the walls of the container in a manner which avoids contact with the cells. Thus the tube does not present side walls so close that there is any significant probability of contact between those cells being sorted and the walls of the tube. In this manner, in addition to the collector fluid (17), it may be desirable to include a wide collection tube as well. Perhaps merely providing a wide opening to the container which serves as part of the collector (14) may be sufficient. For applications utilizing high speed sorting of sperm cells, it has been found that providing a container having an inner diameter opening of at least 15 millimeters is believed to be sufficient. Specifically when utilizing a 14 ml Falcon test tube in such an application, minimal physical damage to the cells as a result of the collector (14) has been discovered.

It should be noted that even the 14 ml Falcon test tube may not be optimum. Specifically, it is believed that designing a collection container which matches the geometry of the stream (that is, a "stream-matched container") may be most optimal. This stream-matched container may have any or all of the following characteristics: a relatively wide orifice, an elliptically shaped orifice, a lesser height to width ratio than currently involved, an angled or otherwise coordinated presentation such as may present side walls which are parallel to the falling streams, and the like. It may also be desirable to provide a mounting element such as a movable element or medium like ball bearings or the like to permit variable orientation of the tube to match the falling stream desired to be collected. In addition, the physical characteristics for the class of containers such as the existing tube (described as a "Falcon-type" test tube) may include not only the width of the tube but also the material (such polystyrene to which the cells do not stick) out of which it is made and the like. (These material options are well known for the 14 ml Falcon tube.) Thus the container and it collection fluid may

also serve as a cushioning element to minimize physical damage to the cells. It also can serve, by its size, to facilitate collection of adequate numbers of sperm without a significant dilution effect.

Another aspect of the collector fluid (17) can be the fact that it, too, may serve to minimize chemical stresses upon the cells. In one regard, since it may be important to provide a nutrient to the cells both before and after sorting, the collector fluid (17) may be selected so as to provide a coordinated level of nutrient so that the levels are balanced both before and after sorting. For bovine sperm in which a nutrient of egg yolk citrate is utilized at a two percent egg yolk level, it has been discovered that utilizing a six percent egg yolk citrate level (that is six percent egg yolk content in a citrate solution) provides good results. This is as result of the volumes existing before and after the sorting event. The collector fluid (17) may start (before sorting) with about 2 ml of volume. The sorting event may add about double this volume (ending at three times the initial starting volume) with very little egg yolk citrate in solution (due to clogging and other flow cytometer considerations). Thus, the end result in terms of the level of the amount of egg yolk citrate present may be equivalent to the starting result, namely, two percent egg yolk content in a citrate solution due to the volumes involved. Thus the collector fluid (17) may be selected so as to create an ending collector fluid environment which is balanced with the initial nutrient or other fluid environment. In this manner, it may serve to minimize the time and changed level of composition to which the cells are subjected. Naturally, these fluid environments may be presented within the flow cytometer or may exist at some other prior time, the important point being merely minimizing the stress to which the cells are subjected at any time in their life cycle. Furthermore, since the initial chemical substance content can be varied (for instance the percent egg yolk content in the citrate may be varied up or down), likewise the starting collection fluid environment or various volumes may also be varied so that the ending result is the same. Thus, prior to commencing the sorting process, the collector fluid exists with a six percent egg yolk content in the citrate solution and after completion of the sorting event the collector fluid—with the sex-specific sperm—may result in a two percent egg yolk content in the citrate solution similar to the initial nutrient content.

time. In the equine species the sample may then be used in oviductal or other insemination processes as those skilled in the art well understand. In bovine sperm, the sample may be diluted yet one more time to the desired dosage level. It has been discovered that dilution may create an effect upon the sperm cell's viability and so it may be appropriate to avoid too large a level of dilution by providing a smaller sample. At present, low dosages of approximately 300,000 sperm per 0.184 ml may be achieved. Furthermore, it may be desirable to maintain a level of seminal plasma at approximately a five percent level, although the results of this requirement are, at present, mixed. The sperm cell specimen may then be placed in a straw for use in artificial insemination and may be transported to the cows or heifers to be inseminated

In order to achieve conveniently timed artificial insemination, heifer or cow estrus may be synchronized using known techniques such as the utilization of prostaglandin $F2_{\alpha}$ according to techniques well known in the art. This latter substance may be particularly valuable in that it has been reported to potentially achieve enhanced fertility in heifers as discussed in the article "Prostoglandin $F2_{\alpha}$ - A Fertility Drug in Dairy Cattle?", 18 Theriogenology 245 (1982) hereby incorporated by reference. While recent results have not maintained this premise, it may be that the present invention demonstrates its particular viability in situations of sexed, low dose insemination. For bovine species, artificial insemination may then be accomplished through the use of embryo transfer equipment with placement of the sperm cells deep within the uterine horns. This may be accomplished not at the peak moment as typically used in artificial insemination, but rather at a somewhat later moment such as 12 hours after that time since there is some possibility that fertility for sexed artificial insemination may occur slightly later. The utilization of embryo transfer equipment may be used because there may be high sensitivity of the uterine wall for such low dose, sexed inseminations.

Interestingly, rather than inseminating within the uterine body where such insemination are usually placed, by insemination deep within the uterine horn, better results may be achieved. Perhaps it is also surprising that the samples thus far studied have shown no difference between ipsi- and contra-lateral inseminations when accomplished deep within the uterine horn. By deep, it should be understood that the insertion is placed well into the uterine horn using the embryo transfer equipment.

The fact that results do not appear significantly different using ipsi- and contra-lateral inseminations has led the present inventors to propose the use of insemination in both so that the process of identifying the appropriate uterine horn may no longer be needed.

As a result of the insemination, it is of course desired that an animal of the desired sex be produced. This animal may be produced according to the systems discussed earlier through the use of the sexed sperm specimen. It should also be understood that the techniques of the present invention may find application in other techniques such as laproscopic insemination, oviductal insemination, or the like.

As examples, the following experiments have been conducted. While not all use every aspect of the inventions described here, they do show the performance enhancements possible through differing aspects of the invention. Further, a summary of some experiments is contained in the article "Uterine Horn Insemination of Heifers With Very Low Numbers of Non-frozen and Sexed Spermatozoa" as referenced earlier. This article summarizes some of the data showing the efficacy of the present invention. As to the experiments, one has been conducted with sexed, unfrozen sperm cells with high success as follows:

EXAMPLE 1

Angus heifers, 13-14 mo of age and in moderate body condition, were synchronized with 25 mg of prostaglandin F-2 alpha at 12-day intervals and inseminated 6-26 h after observed standing estrus. Freshly collected semen from three 14-26 mo old bulls was incubated in 38 μM Hoechst 33342 at 75 x 10^6 sperm/ml in a TALP medium for 1 h at 34°C. Sperm were sorted by sex chromosomes on the basis of epiflourescence from laser excitation at 351 and 364 nm at 150 mW using a MoFlo® flow cytometer/cell sorter operating at 50 psi and using 2.9% Na citrate as sheath fluid. X chromosome-bearing sperm (~90% purity as verified by resorting sonicated sperm aliquots) were collected at ~500 live sperm/sec into 2-ml Eppendorf tubes containing 100 μl Cornell Universal Extender (CUE) with 20% egg yolk. Collected sperm were centrifuged at 600 x g for 10 min and resuspended to 1.63 x 10^6 live sperm/ml in CUE. For a liquid semen unsexed control; Hoechst 33342-stained sperm were diluted with sheath fluid to 9 x 10^5 sperm/ml and centrifuged and resuspended to 1.63 x 10^6 progressively motile sperm/ml in CUE. Sexed semen and liquid control semen were cooled to 5°C over 75 min and loaded into 0.25-ml straws (184 ul/straw). Straws